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Local Immune Response in Experimental Pyelonephritis in the Rabbit

I. MORPHOLOGICAL AND FUNCTIONAL FEATURES OF THE LYMPHOCYTIC INFILTRATE

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Summary. The cellular activity of circulating lymphocytes and lymphocytes isolated from the infected kidney of animals with experimental haematogenous pyelonephritis was evaluated. The incorporation of [^{3}H-methyl]thymidine into DNA by lymphocytes was studied with mitogens such as phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and goat anti-rabbit IgG (GARIG). Lymphocytes from infected kidney had a high baseline DNA synthesis compared to circulating lymphocytes from days 5 to 27 of infection. Infected kidney lymphocytes failed to respond to PHA, PWM, or GARIG, whereas circulating lymphocytes did respond to these mitogens. Uropod-bearing lymphocytes, which were shown to be T lymphocytes, were present from days 5 to 77 of infection. B lymphocytes, as determined by surface immunofluorescent technique, were present by day 12, coincident with the onset of local synthesis of antibody. These studies reveal that in pyelonephritis, the cellular response goes through sequential changes and indicate a dynamic interrelationship between T and B lymphocytes at an infected site.

INTRODUCTION

A characteristic feature of pyelonephritis is an interstitial lymphocytic cellular response. One expression of this response in experimental pyelonephritis is the local synthesis of immunoglobulin and antibody (Lehmann, Smith, Miller, Barnett and Sanford, 1968). The principal immunoglobulin is IgG, but significant quantities of secretory IgA are also synthesized. Antibody against the infectious agent is present by day 11 and persists for at least 150 days after the onset of the infection (Lehmann *et al.*, 1968; Smith, Holmgren, Ahlstedt and Hanson, 1974). However, little information is available on the cellular immune response in the kidney in pyelonephritis. Previously reported studies in pyelonephritis have examined the cellular function of either circulating lymphocytes in response to the infecting antigen in humans with pyelonephritis or lymphocytes from lymph nodes of animals with pyelonephritis (Soltys and Brody, 1968; Montgomerie, Kalmanson and Guze, 1969). The purposes of the following study were to examine the activity of lympho-

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cytes from infected kidneys, and to evaluate isolated lymphocytes for T-cell and B-cell surface characteristics.

MATERIALS AND METHODS

Isolation of lymphocytes

Unilateral pyelonephritis was produced in male New Zealand white rabbits as previously described (Lehmann *et al.*, 1968). The right ureter was transiently obstructed with a polyethylene ligature and 1×10^8 *Escherichia coli* O75 were injected intravenously. The ligature was released after 18 hours. At various times following infection, animals were studied. The right kidney was examined for gross evidence of pyelonephritis. Tissue wedges were removed from infected kidneys, fixed in 10 per cent formalin, sectioned, and stained with haematoxylin and eosin. Tissue fragments were obtained for recovery of single lymphocytes and study of *in vitro* protein and immunoglobulin synthesis.

Tissue was teased through number 50 stainless steel wire mesh using a haemostat and wire mesh scrapers (Swenson and Kern, 1967). The isolated lymphocytes were handled in tissue culture media containing minimum essential media (MEM), or Roswell Park Memorial Institute (RPMI) 1640 (Grand Island Biological Laboratories, Grand Island, New York), penicillin, streptomycin and glutamine. Circulating lymphocytes were obtained by intracardiac puncture using 30-ml syringes containing 100 units of sodium heparin and 5 ml of sterile 6 per cent solution of dextran in normal saline. The blood–dextran mixture was allowed to settle at 3° for 1 hour, and the leucocyte-rich plasma was filtered through glass wool (Douglas, Kamin and Fudenberg, 1969), packed in syringes and the cells were washed with tissue culture media. Cells were centrifuged at 400 ***g*** for 10 minutes in a refrigerated centrifuge at 4° and washed twice in 10 ml of tissue culture media. Circulating and kidney lymphocytes were resuspended in media, and a cell count performed in a Neubauer chamber. Methylene blue staining of the lymphocyte preparation revealed less than 5 per cent of cells to be polymorphonuclear. To evaluate for monocytes, cells were incubated with latex particles and examined for phagocytosis (Zucker-Franklin, 1974). Trypan blue staining revealed viability exceeding 98 per cent in all preparations. Lymphocytes from lymph nodes of normal rabbits from popliteal region were obtained by teasing through wire mesh.

DNA synthesis

Cells were adjusted to a final concentration of 2×10^6 cells per millilitre in RPMI 1640 containing 8 per cent rabbit serum decomplemented by heating to 56° per 1 hour and incubated in triplicate. Tubes in triplicate were also incubated with either 0·1 ml of a 1:5 dilution of phytohaemagglutinin-M (PHA) (Difco, Detroit, Michigan) (final concentration of 1:60), 1:60 dilution of pokeweed mitogen (PWM), or 0·1 ml of goat anti-rabbit IgG (GARIG) in each tube at 37° in a 5 per cent CO_2 incubator for 3 days. Eighteen hours prior to the termination of the culture, two microcuries of [^{3}H-methyl]thymidine (Schwarz-Mann Bio-Research, Orangeburg, New York) (specific activity 1·9 Ci/mmole) was added to each tube. At the end of 3 days, the cell suspension was spun and processed as described by Newberry and Sanford (1971). The cells were centrifuged at 4° at 800 ***g*** for 10 minutes and washed twice with cold phosphate-buffered saline. The cells were then precipitated with 3 ml of cold 7 per cent trichoroacetic acid (TCA), centrifuged at 800 ***g*** for 10 minutes, and washed once with cold 7 per cent TCA. The precipitate was digested

in 0·5 ml of 2 N sodium hydroxide for 1 hour. An aliquot of the digested material was transferred to a test tube containing 2 N sodium hydroxide, and Beckman Biosolv–2 (BBS2) (Beckman Instruments, Fullerton, California) was added to achieve a neutral pH. The mixture was then added to 10 ml of a scintillation cocktail containing toluene-2, 5, diphenyloxazole (PPO), 7 g/l. Liquid scintillation counting was performed in a Beckman LS-250 Spectrometer. The uptake of [^{3}H]thymidine into DNA is expressed as counts per minute (ct/minute) per 2×10^6 lymphocytes.

In certain experiments, infected kidney tissue was studied to see if a soluble factor (blastogenic factor) was present which would lead to enhanced DNA synthesis by peripheral blood leucocytes (Janis and Bach, 1970). The supernatant obtained following the first centrifugation of the kidney lymphocytes was used to resuspend circulating lymphocytes from the same animal. Incubation was carried out for 3 days in the presence of 8 per cent normal rabbit serum. Uptake of tritiated thymidine was determined on the circulating lymphocytes in the presence of this supernatant and compared to baseline activity in tissue culture media as described above.

Preparation of lymphocytes for uropod examination

Circulating lymphocytes and cells from infected kidney were incubated at 37° for 30 minutes at a concentration of 5×10^6 lymphocytes per millilitre in RPMI containing 10 per cent normal rabbit serum. Cell suspensions were incubated with latex particles so that it would be possible to distinguish lymphocytes from monocytes. Cells were transferred to a glass slide warmed to 37° for 15–60 minutes. The cells were then examined with a phase contrast microscope (Carl–Zeiss, Oberkochen, West Germany), using a microscope slide incubator to maintain cell temperature at 37°. The percentage of uropod-bearing lymphocytes to lymphocytes was determined on the basis of examination of between 150–300 lymphocytes (Rosenstreich, Shevach, Green and Rosenthal, 1972).

Surface immunoglobulin determination

Antibodies to rabbit immunoglobulin classes M and G were eluted from immunoabsorbent columns containing the homologous immunoglobulin and were made specific by passage over immunoabsorbent columns of Sepharose 4B coupled with immunoglobulins of the classes to which it was not directed. The purified antibodies at a concentration of 10 mg/ml were adjusted to pH 9·5 by addition of sodium carbonate and conjugated with fluorescein isothiocyanate (FITC) added in a proportion of 10 μg/mg of protein. Conjugation proceeded at room temperature for 2 hours and then non-conjugated FITC was removed by passage over a Sephadex G-25 column equilibrated with 0·1 M phosphate buffer, pH 6·8. FITC conjugates with fluorescein to protein (F/P) ratio of 3·0 to 4·0 were used to examine for surface immunoglobulins. Circulating and kidney lymphocytes from normal and infected animals were incubated for 20 minutes at 4° with fluorescent antibodies at concentrations of 0·4 mg/ml in RPMI 1640 (Lawton Asofsky, Hylton and Cooper, 1972). The cells were washed once through a gradient of foetal calf serum, once with RPMI 1640 and then placed on slides. A coverslip was applied and sealed with nail polish. Lymphocytes were examined with a Leitz microscope equipped with an HBO 200 mercury arc lamp, BG12 excitation filters and a 500 barrier filter.

Protein immunoglobulin and specific antibody synthesis

The technique for determination of protein, immunoglobulin and anti-*E. coli* O75

specific antibody synthesis by the incorporation of [^{14}C]amino acids into protein has been described previously in detail (Lehmann *et al.*, 1968; Hand, Smith, Miller, Barnett and Sanford, 1971; Miller, Smith and Sanford, 1971). Newly synthesized immunoglobulin was quantified by precipitation in antibody excess with goat anti-rabbit IgG (GARIG), GAR–colostral IgA and GAR–IgM. Synthesis of IgG antibody against infecting organism was determined by measuring the binding of ^{14}C to a heat-killed suspension of *E. coli* O75, using *Pseudomonas aeruginosa* as a control antigen.

RESULTS

MICROSCOPIC APPEARANCE OF CELLULAR INFILTRATE OF INFECTED KIDNEY

Approximately 70 per cent of lymphocytes isolated from kidneys of normal animals were medium-sized lymphocytes and 30% small lymphocytes. In pyelonephritic kidneys, the predominant mononuclear cells up to day 13 were medium to large-sized lymphocytes with thin cytoplasm. Less than 5 per cent of the cells ingested latex particles, indicating that over 95 per cent of cells were functionally lymphocytes. Only a few darkly staining small lymphocytes were present. By the 18th day of infection, approximately 50 per cent were small lymphocytes and by day 35 more than 80 per cent of cells were small, densely stained lymphocytes. In microscopic sections, these lymphocytes often accumulated in dense clusters resembling follicles from the cortex of a lymph node.

DNA SYNTHESIS

Lymphocytes obtained from the kidneys of normal animals had a variable activity of DNA synthesis (Table 1). Some animals had a low baseline, whereas lymphocytes from other kidneys had a high baseline DNA synthesis. Lymphocytes from normal kidney showed no increase in DNA synthesis after exposure to PHA. Circulating lymphocytes from normal animals demonstrated low DNA synthesis (mean of 820 ct/minute per 2×10^6 lymphocytes) which increased significantly in the presence of PHA (mean increase of 179-fold). Lymph node lymphocytes had a much higher baseline synthesis of DNA (mean of 10,980 ct/minute) than did circulating cells, which increased significantly (eight-fold) with PHA.

TABLE 1

DNA SYNTHESIS BY CELLS FROM NORMAL ANIMALS

Normal kidney			Lymph node			Circulating lymphocytes		
Baseline	PHA*	Increase	Baseline	PHA*	Increase	Baseline	PHA*	Increase
9710	6480	0·7	21,000	87,375	4·2	1535	128,590	83
860	430	0·5	—	—	—	500	145,695	291
430	320	0·7	1125	63,990	57	—	—	—
—	—	—	—	—	—	345	111,920	324
4935	1145	0·3	10,815	123,040	11	900	200,360	223
Mean ± s.e.								
3983	2168		10,980	91,458		820	146,641	
± 1870	± 1263	(× 0·5)	± 4688	± 14,020	(× 8)	± 230	± 16,630	(× 179)

The results are expressed as [^{3}H]thymidine uptake (ct/minute) by 2×10^6 lymphocytes.

* Cells incubated in presence of concentration of 1:60 dilution of phytohaemagglutinin-M.

Cells isolated from the pyelonephritic kidney early in infection had a high uptake of tritiated thymidine (Table 2, Fig. 1). PHA failed to stimulate kidney cells in contrast to the response of circulating and lymph node lymphocytes. A high baseline activity of DNA synthesis was present up to day 27, although variation occurred from animal to animal. By day 35 the cells showed very little synthesis of DNA. Viable bacteria for the infecting organism, *E. coli* O75 were only present consistently in the kidney up to day 11, although enhanced DNA synthesis by infected kidney lymphocytes was noted up to day 27. Lymphocytes obtained from circulating blood resembled normal circulating lymphocytes in having a low baseline activity of DNA synthesis and a significant increase with PHA (seventeen-fold mean increase). Although circulating lymphocytes from normal animals showed a significant increase in DNA synthesis in the presence of PWM (116-fold) and GARIG (seventeen-fold), lymphocytes from pyelonephritic kidney failed to respond to either of these mitogens.

TABLE 2

DNA SYNTHESIS BY LYMPHOCYTES FROM ANIMALS WITH PYELONEPHRITIS

		Pyelonephritic kidney			Circulating lymphocytes		
Day of infection	Bacterial culture right kidney	Baseline	PHA*	Increase	Baseline	PHA*	Increase
4	+	6830	2315	0·3	2005	5595	2·8
9	+	1990	1595	0·8	140	1670	12
11	+	3260	3485	1·1	—	—	
12	0	1390	305	0·2	—	—	
13	0	700	730	1·0	385	11,150	29
18	0	3710	3485	0·9	—	—	
19	+	1195	570	0·5	—	—	
20	0	3120	1150	0·4	—	—	
25	0	780	440	0·6	300	27,435	91
27	0	1895	690	0·4	—	—	
35	0	195	160	0·8	880	11,150	13
40	0	295	120	0·4	95	9250	97
Mean ± s.e.	Days 4–27	2490 ± 560	1480 ± 370	(×0·6)	630 ± 270	11,040 ± 4235	(×17)
	Days 35–40	245 ± 50	140 ± 20	(×0·6)			

The results are expressed as [^{3}H]thymidine uptake (ct/minute) by 2×10^6 lymphocytes.

* Cells incubated in presence of 1:60 dilution of phytohaemagglutinin-M.

Supernatants obtained from infected kidney tissue were also examined in four animals to determine if soluble factors were present which would lead to blastogenesis of circulating lymphocytes from the infected animal. No increase in DNA synthesis over baseline was noted in the presence of tissue supernatants.

UROPOD FORMATION

Circulating and kidney lymphocytes obtained from normal animals had no uropod formation (Table 3). By day 5 of infection, significant numbers of uropods (Fig. 2) were found both in circulating lymphocytes and in lymphocytes isolated from the kidney. Uropod formation was noted in circulating lymphocytes up to day 19 whereas uropod-

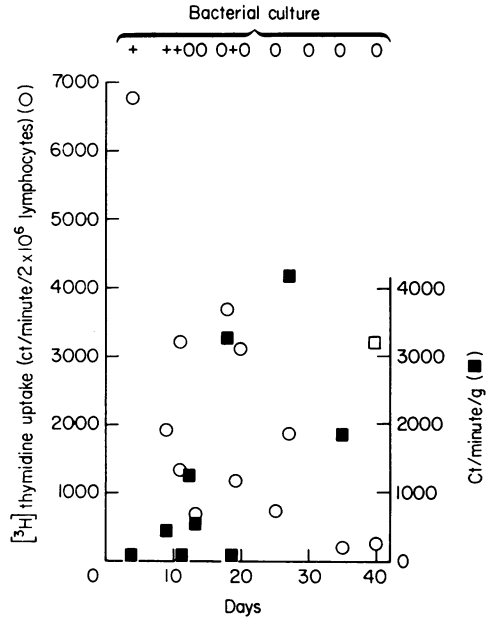

FIG. 1. Comparison of DNA synthesis and specific antibody synthesis by pyelonephritic kidneys. DNA synthesis of lymphocytes from infected kidneys is expressed as uptake of [^{3}H]thymidine by 2×10^6 lymphocytes (○). Specific antibody synthesis is the quantity of [^{14}C]protein from DEAE fractions containing IgG that attaches to heat-killed *E. coli* O75, expressed as ct/minute/g of infected tissue.

TABLE 3

UROPOD FORMATION BY LYMPHOCYTES IN PYELONEPHRITIS (PERCENTAGE OF LYMPHOCYTES WITH UROPODS)

Day of infection	Number	Pyelonephritic kidney	Circulating lymphocytes
Normal	(2)	0	0
5		8	18
12		10	3
12		12	8
16		7	7
19		12	9
21		14	0
27		10	—
27		2	0
32		0	0
33		1	4
40		1	0

bearing lymphocytes persisted from pyelonephritic kidneys up to day 27. Very few uropod-bearing lymphocytes were detected after day 27 in either circulating or kidney lymphocytes. In separate experiments, the fraction of lymphocytes with uropod formation from both circulating and pyelonephritic kidney lymphocytes studied at day 13 was nearly doubled after selective killing of B lymphocytes with anti-immunoglobulin antisera.

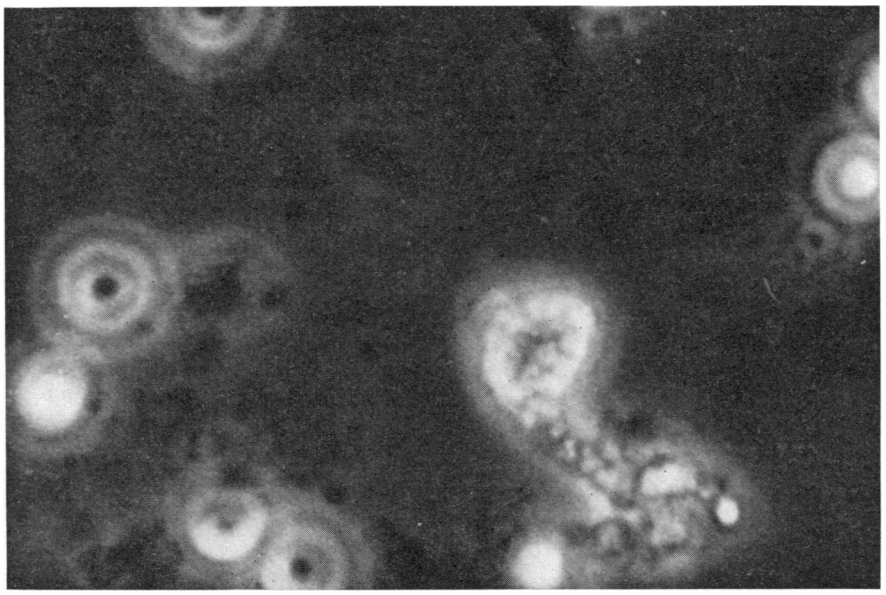

FIG. 2. Photograph of uropod-bearing lymphocyte from infected kidney. (Magnification × 420.)

SURFACE IMMUNOGLOBULIN DETERMINATION OF CIRCULATING AND KIDNEY LYMPHOCYTES

An increase in circulating lymphocytes with surface immunofluorescence was noted in animals with pyelonephritis (Table 4). The increase occurred principally in cells staining for IgM. Few (less than 2 per cent) cells from normal kidney were positive for either IgM or IgG. A small number were present by day 8 of infection, but slightly more than one-third of cells were positive from day 12 up to day 19. A smaller number were positive on day 20. In experiments after day 20, few lymphocytes were obtained and too few of those examined were positive by membrane fluorescence for valid percentages to be tabulated. Incubation of cells with unconjugated antisera abolished staining with homologous fluorescein conjugate.

TABLE 4

PERCENTAGE OF CIRCULATING AND KIDNEY LYMPHOCYTES WITH SURFACE IMMUNOGLOBULINS

	Circulating lymphocyte			Kidney lymphocytes		
	IgM	IgG	Total	IgM	IgG	Total
Days						
Normal	13	27	40	1	1	2
Normal	8	25	33	1·5	0	1·5
Day of infection						
8	30	29	59	4	6	10
12	—	—	—	27	10	37
13	24	34	58	21	18	39
14	—	—	—	18	19	37
19	36	26	62	11	25	36

IMMUNOGLOBULIN AND ANTIBODY SYNTHESIS OF PYELONEPHRITIC KIDNEY

Significant local immunoglobulin and antibody synthesis was noted with pyelonephritis. Tissues obtained from animals early (days 4–9) in the course of pyelonephritis showed only a slight increase in immunoglobulin synthesis compared to normal (Table 5). In animals studied from days 11 to 27, a significant increase in immunoglobulin (principally IgG) occurred. As shown previously by day 35 IgA synthesis was present in significant quantities (Smith, Hand and Sanford, 1972). The synthesis of IgM was only slightly increased above control values. Significant synthesis of antibody against the infecting organism was present by day 11 and persisted up to day 40 (Fig. 1). Synthesis of specific antibody reached a high level and persisted as baseline DNA synthesis by lymphocytes diminished.

TABLE 5

IMMUNOGLOBULIN SYNTHESIS BY PYELONEPHRITIC KIDNEY (mean ct/minute/g ± s.e.m.)

Days	(Number studied)	Total protein	IgG	IgA	IgM	Specific antibody (IgG)	Percentage of total protein		
							IgG	IgA	IgM
Normal*	(6)	2650 ± 330	90 ± 20	120 ± 20	40 ± 20	0	4	4	1
4–9	(3)	18,910 ± 8000	3230 ± 1870	510 ± 180	390 ± 150	220	17	3	2
11–27	(8)	39,120 ± 12,890	24,760 ± 14,510	1090 ± 610	800 ± 500	1580	63	3	2
35–40	(2)	50,120	28,025	5050	650	2520	56	10	1

DISCUSSION

In these studies of experimental pyelonephritis, lymphocytes at the infected site change both in morphological appearance and in functional activity. Lymphocytes from normal kidney show variable DNA synthesis and differ from circulating lymphocytes or lymph node lymphocytes by failing to divide in the presence of phytohaemagglutinin (PHA) (Daguillard and Richter, 1969). Since these tissue lymphocytes do not have surface membrane markers for immunoglobulins, they may represent 'null' cells (Williams, DeBoard, Mellbye, Messner and Lindstrom, 1973). Early in infection, lymphocytes which initially accumulate in the infected kidney are medium-sized cells, are active in synthesizing DNA, and are uropod-bearing lymphocytes. Later in infection, small lymphocytes which synthesize little DNA predominate.

Uropod formation was selected as a marker for activated cell and for T lymphocytes. Rosenstreich *et al.* (1972) showed that uropod-bearing lymphocytes in guinea-pigs were T lymphocytes since they had no surface immunoglobulins, and selective removal of B lymphocytes increased the percentage of uropod-bearing lymphocytes. However, de Petris and Raff (1972) demonstrated membrane-bound immunoglobulin resides on lymphocytes which they described as forming uropods. In our studies, uropod-bearing lymphocytes were increased by selective removal of B lymphocytes, also suggesting that these cells are T lymphocytes. In pyelonephritis, the initial appearance of uropod-bearing lymphocytes coincided with the period of enhanced DNA synthesis by kidney lymphocytes, and they persisted during the period when significant antibody production was detected. Uropod-bearing lymphocytes appear to be activated cells, which have been observed in mixed

leucocyte reactions, and to interact with macrophages, cell debris, and lymphoblasts and make cell-to-cell contact between lymphocyte and target cells (McFarland and Heilman, 1965; McFarland, 1969; Biberfield, 1971).We postulate that these cells represent activated cells which function as helper cells, and interact with antigen, thus working in a co-operative manner with (B) lymphocytes in pyelonephritis. We have attempted to detect the release of soluble mediators such as lymphotoxin or MIF by these activated cells, and have failed to detect any activity. These findings, although negative, indicate that T lymphocytes present in infected kidney may not react in a typical manner (Bloom, and Bennett, 1970) but rather represent a subpopulation of activated T lymphocytes. Recently, Miller, Simpson and Ormrod, (1975) have suggested that kidney cells may affect the response of T lymphocytes to mitogens in pyelonephritis. Studies by Coles *et al.* (Coles, Chick, Hopkins, Ling and Radford, 1974) have indicated that the course of pyelonephritis is the same after removing T cells prior to infecting rats, which suggests that T cells may function as helper cells.

Following the appearance of these active T lymphocytes on day 12, B lymphocytes, appear in significant number. Their appearance coincides with the synthesis of IgG and specific antibody. These small lymphocytes form a community resembling cortical nodules of lymph nodes, which are known to contain B lymphocytes. This anatomical association of lymphocytes may explain why more immunoglobulin and antibody synthesis occurs when tissue fragments are incubated than when single cells are cultured (Smith *et al.*, 1972). We failed to detect a sequence of surface-bearing lymphocytes of IgM to IgG cells as has been noted ontogenetically by Lawton *et al.* (1972). Recently, we have utilized the more sensitive enzyme-linked immunoabsorbent assay, and have shown that antibody in the IgG class precedes IgM antibodies in pyelonephritis (Smith *et al.*, 1974). These studies emphasize the different features of the immune response at a local site of infection compared to previous observations based on circulating cells or serum antibody. We also noted in these studies an increase in the proportion of circulating B lymphocytes in infected animals. A similar significant increase in B lymphocytes have been noted in humans with bacterial infections (Thorley, Smith, Luby and Sanford, 1975).

Thus, these studies demonstrate a sequential change in the morphology and function of lymphocytes at an infected site. Other than residual antigen, it is not known what promotes the rapid accumulation of active lymphocytes. Attempts to see if factors present in the inflammatory tissue supernatants would induce blastogenesis (Janis and Bach, 1970) were unsuccessful. Also of interest, they were actively synthesizing DNA up to 2 weeks after organisms could no longer be cultured. Although the origin of the kidney lymphocyte infiltrate is not readily apparent, it seems probable that they are derived from circulating lymphocytes and that early in pyelonephritis, T cells accumulate followed shortly by B lymphocytes.

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